Supporting Information

Sysoeva et al. 10.1073/pnas.1322200111

SI Materials and Methods

Construction of Strains and Growth Conditions. Plasmids were constructed and maintained using standard molecular cloning protocols and *Escherichia coli* strain DH5α (1). All changes to the YukE substrate were introduced by site-directed mutagenesis of either *Bacillus subtilis* integration or *E. coli* expression vectors and confirmed by sequencing. Strains are listed in Table S1, and oligonucleotides used in this work are summarized in Table S2. For secretion assays and analyses of cellular content, *B. subtilis* strains were grown in LB medium supplemented with an inducer at specified concentrations when needed. The antibiotics ampicillin (100 μg/mL), kanamycin (5 or 50 μg/mL for *B. subtilis* and *E. coli*, respectively), spectinomycin (100 μg/mL), and erythromycin (MLS) (1 μg/mL) plus lincomycin (25 μg/mL) were included when appropriate. *B. subtilis* strains were created by transforming domesticated strain PY79 by natural competence with the listed plasmid for double crossover (2).

Cloning, Expression, and Purification of the Modified YukE Variants. The coding sequence of YukE was cloned into pET28b+ vector (Novagen) and *B. subtilis* into integration vector pDR111 [gift of D. Rudner (Harvard Medical School, Boston)]. Recombinant protein for purification was produced by overexpression in *E. coli* strain BL21(DE3) upon induction in LB medium with 0.5–1 mM isopropyl β-D-1-thiogalactopyranoside at an OD_{600} of ∼0.6 for 16 h at 30 °C. The cells were lysed using a TS series Cell Disruptor (Pressure Biosciences Inc.) at 17,000 psi in lysis buffer [50 mM Hepes pH 7.5, 250 mM NaCl, 5% (wt/vol) glycerol] supplemented with a complete protease inhibitor mixture tablet (Roche). Protein was purified from the cell lysate using nickel affinity chromatography in lysis buffer and elution into the same buffer supplemented with 0.5 M imidazole. Eluted YukE protein was then dialyzed into 25 mM Hepes pH 7.5, 20 mM NaCl, 5% (wt/vol) glycerol. When required, hexahistidine tag was cleaved with thrombin overnight at 25 °C during dialysis (3 MWCO tubing, Pall) into an appropriate buffer, leaving extra Gly–Ser–His residues. The thrombin was captured by incubating the preparation with p-Aminobenzamidine–Agarose (Sigma-Aldrich) in the same buffer.

1. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).

2. Harwood CR, Cutting SM, eds (1990) *Molecular Biological Methods for Bacillus* (Wiley, New York).

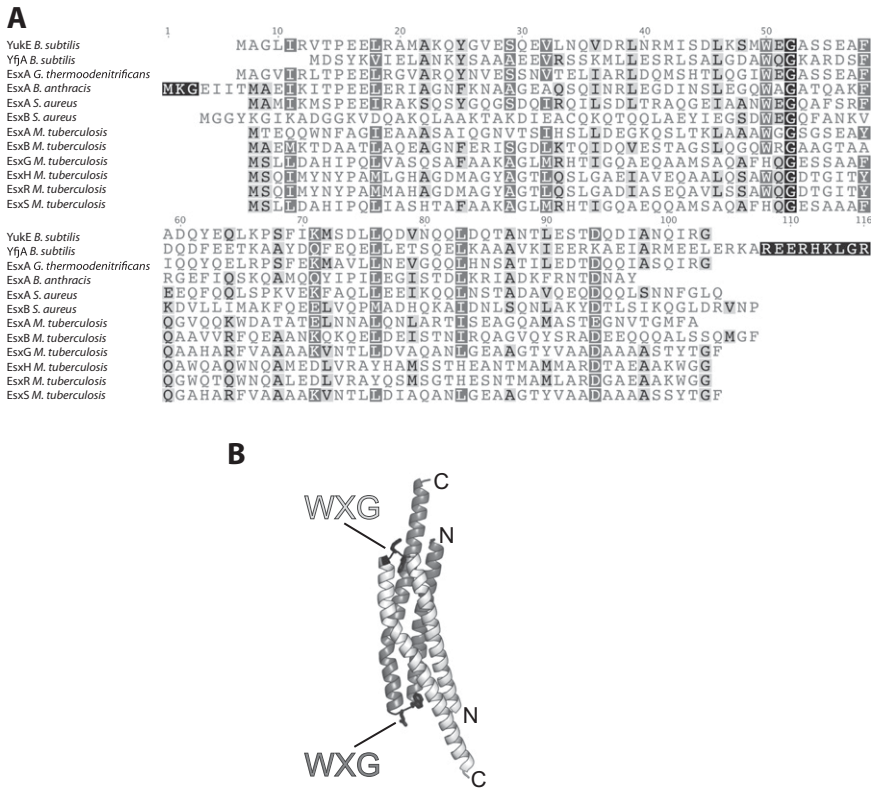


Fig. S1. Substrates of the ESX secretion systems–WXG100 proteins. (*A*) Sequence alignment of YukE substrate of *B. subtilis* and other WXG proteins including prototypical ESX substrates EsxA (ESAT-6) and EsxB (CFP-10) of *Mycobacterium tuberculosis*. (*B*) WXG proteins possess a conserved helix-turn-helix fold and form stable dimers. The 3D model of YukE monomer in cartoon representation with the defining WXG motif is given in black stick representation. The model was built in Swiss-PDBViewer (1, 2) via threading of the YukE sequence to the structure of EsxA from *Geobacillus thermodenitrificans* [Protein Data Bank (PDB) ID code 3ZBH]. Structural illustrations in this and other figures are prepared using PyMOL software (The PyMOL Molecular Graphics System, Version 1.5.0.4, Schrödinger, LLC).

1. Guex N, Peitsch MC (1997) SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. *Electrophoresis* 18(15):2714–2723.
2. Guex N, Peitsch MC, Schwede T (2009) Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: A historical perspective. *Electrophoresis* 30(Suppl 1): S162–S173.

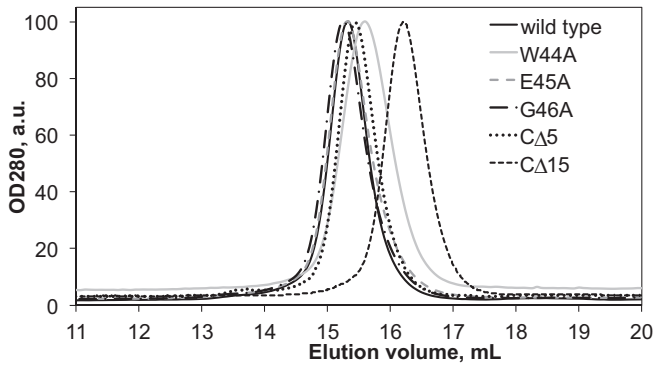
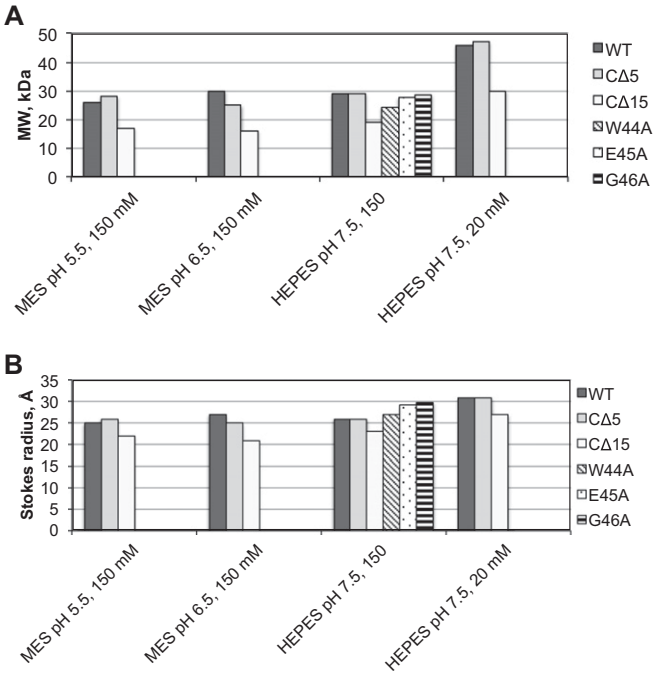


Fig. S2. WXG-substituted and C-terminally truncated YukE variants form dimers in vitro. Size-exclusion chromatography (SEC) analyses of recombinant YukE substrates. Recombinant wild-type and mutated YukE proteins were analyzed on a Superdex200 column that was calibrated using BioRad gel filtration standards. The estimated molecular weights and Stokes radii are given in Fig. S3.



C

	monomer	dimer	tetramer	MES pH 5.5, 150 mM NaCl	MES pH 6.5, 150 mM NaCl	HEPES pH 7.5, 150 mM	HEPES pH 7.5, 20 mM
	MW calculated, kDa			**MW estimated, kDa**			
WT	11	23	45	26	30	29	46
CΔ5	11	21	43	28	25	29	47
CΔ15	10	19	39	17	16	19	30
W44A	11	23	45	-	-	24	-
E45A	11	23	45	-	-	28	-
G46A	11	23	45	-	-	29	-
	Rs calculated, Å			**Rs estimated, Å**			
WT	20	24	32	25	27	26	31
CΔ5	19	23	30	26	25	26	31
CΔ15	17	21	28	22	21	23	27
W44A	20	24	32	-	-	27	-
E45A	20	24	32	-	-	29	-
G46A	20	24	32	-	-	30	-

Fig. S3. In vitro dimerization of recombinant YukE protein under different pH and salt conditions. (*A*) Estimating molecular weights of YukE variants using SEC. YukE remains in the dimeric form in the tested pH range from 5.5 to 7.5 (buffer is indicated; NaCl is 20 mM or 150 mM). At low-salt conditions, YukE species shifts to a higher apparent molecular weight, indicating that it either is forming complexes with more than just two subunits or is interacting non-specifically with the Superdex resin. (*B*) Estimating Stokes radii of YukE variants via SEC. Theoretical Stokes radii were estimated from the 3D models of YukE threaded on the crystal structure of *G. thermodenitrificans* EsxA (PDB ID code 3ZBH) in SOMO software (UltraScan Program Suite) (1–3). (*C*) Summary of the SEC parameters.

1. Brookes E, Demeler B, Rocco M (2010) Developments in the US-SOMO bead modeling suite: New features in the direct residue-to-bead method, improved grid routines, and influence of accessible surface area screening. *Macromol Biosci* 10(7):746–753.
2. Brookes E, Demeler B, Rosano C, Rocco M (2010) The implementation of SOMO (SOlution MOdeller) in the UltraScan analytical ultracentrifugation data analysis suite: Enhanced capabilities allow the reliable hydrodynamic modeling of virtually any kind of biomacromolecule. *Eur Biophys J* 39(3):423–435.
3. Rai N, et al. (2005) SOMO (SOlution MOdeler) differences between X-ray- and NMR-derived bead models suggest a role for side chain flexibility in protein hydrodynamics. *Structure* 13(5):723–734.

Table S1. Summary of tested variants of WXG proteins from different organisms

Substitution	YukE mutant	EsxA	EsxB	YukE	Other effects for YukE homologs
EsxA *M. tuberculosis*					
W43R (1)	W44	+	+	–	Does not pull down EsxB
G45T (1)	G46	+	+	–	
T2H (1)		+	+	n/t	
Q4L (1)		+	+	n/t	
F8I (1)		+	+	n/t	
A14R (1)		+	+	n/t	
L28A/L29S (1)		+	+	n/t	
Q55/Q56A (1)		+	+	n/t	
N66I/N67A (1)		+	+	n/t	
M83I (1)		+	+	n/t	
V90R (1)		+	+	n/t	
M93T (1)		+	+	n/t	
F94Q (1)		+	+	n/t	
CΔ84–96 (1)	CΔ12	+	+	–	
L29D (2, 3)		n/t	n/t	n/t	Does not heterodimerize
G32D (2)		n/t	n/t	n/t	Heterodimerizes
A41D (2)		n/t	n/t	n/t	Heterodimerizes
L65D (2, 3)		n/t	n/t	n/t	Does not heterodimerize
CΔ76–95 (4)		–	–	n/t	Not detectable in cells
NΔ3–24 (4)		–	–	n/t	Not detectable in cells
EsxB *M. tuberculosis*					
S96A (5)		+	+	n/t	Heterodimerizes
F100A (5)		–	–	n/t	Heterodimerizes
M98A (5)		–	–	n/t	Heterodimerizes
CΔ7 (5)		–	–	–	
CΔ25 (5)		–	–	n/t	Not detectable in cells
L94A (5)				n/t	Heterodimerizes
S95A (5)				n/t	Heterodimerizes
Q97A (5)				n/t	Heterodimerizes
G99A (5)				n/t	Heterodimerizes
Y83A (6)	L84A	–	–	n/t	
E87A (6)	D88A	–	–	n/t	
Y83A/E87A (6)	L84A/D88A	–	–	n/t	
W43R (3)	W44	n/t	n/t	n/t	Heterodimerizes
I21R (3)		n/t	n/t	n/t	Heterodimerizes
L25R (3)		n/t	n/t	n/t	Does not heterodimerize
F58R (3)		n/t	n/t	n/t	Does not heterodimerize
EspA *M. tuberculosis*					
W55R (7)	W44R	–	–	–	EspA itself is not secreted
G57R (7)	G46R	–	–	–	EspA itself is not secreted
F5R (7)		–	–		Unstable EspA
K41A (7)		–	–		Unstable EspA
F50R (7)	D39R	–	–	n/t	Unstable EspA; no proper equivalent in YukE
K62A (7)	A51A	–	–	n/t	Unstable EspA; no proper equivalent in YukE
EsxB *Bacillus anthracis*					
CΔ85–90 (8)	CΔ12	n/a	+	–	
CΔ81–90 (8)	CΔ16	n/a	–	n/t	Not stable
NΔ1–6 (8)	n/a	n/a	+	n/t	
NΔ1–11 (8)	NΔ1–4	n/a	–	n/t	Not stable
EsxD *Staphylococcus aureus*					
CΔ6 (9)	n/a	–	+	–	Secretion of EsxD and EsxB is not affected; EsxA and EsxC—not secreted
Y100A, E104A (9)	n/a	+	+	n/t	Esx secretion is not disrupted

n/a, not applicable; n/t, not tested.

1. Brodin P, et al. (2005) Functional analysis of early secreted antigenic target-6, the dominant T-cell antigen of Mycobacterium tuberculosis, reveals key residues involved in secretion, complex formation, virulence, and immunogenicity. *J Biol Chem* 280(40):33953–33959.

2. Meher AK, Bal NC, Chary KV, Arora A (2006) Mycobacterium tuberculosis H37Rv ESAT-6-CFP-10 complex formation confers thermodynamic and biochemical stability. *FEBS J* 273(7): 1445–1462.
3. Meher AK, Lella RK, Sharma C, Arora A (2007) Analysis of complex formation and immune response of CFP-10 and ESAT-6 mutants. *Vaccine* 25(32):6098–6106.
4. Brodin P, et al. (2006) Dissection of ESAT-6 system 1 of Mycobacterium tuberculosis and impact on immunogenicity and virulence. *Infect Immun* 74(1):88–98.
5. Champion PA, Stanley SA, Champion MM, Brown EJ, Cox JS (2006) C-terminal signal sequence promotes virulence factor secretion in Mycobacterium tuberculosis. *Science* 313(5793): 1632–1636.
6. Daleke MH, et al. (2012) General secretion signal for the mycobacterial type VII secretion pathway. *Proc Natl Acad Sci USA* 109(28):11342–11347.
7. Chen JM, et al. (2013) Phenotypic profiling of Mycobacterium tuberculosis EspA point mutants reveals that blockage of ESAT-6 and CFP-10 secretion in vitro does not always correlate with attenuation of virulence. *J Bacteriol* 195(24):5421–5430.
8. Garufi G, Butler E, Missiakas D (2008) ESAT-6-like protein secretion in Bacillus anthracis. *J Bacteriol* 190(21):7004–7011.
9. Anderson M, Aly KA, Chen YH, Missiakas D (2013) Secretion of atypical protein substrates by the ESAT-6 secretion system of Staphylococcus aureus. *Mol Microbiol* 90(4):734–743.

Table S2. Strains used in this study

Name	Genotype	Source
B. subtilis		
PY79	Laboratory strain that was used as wild-type background for all other strains in this work	B.M.B. laboratory
bLH015	*yukE*::*erm*-Pyuk	(1)
bLH045	*yukE*::*erm*-Pyuk; *amyE*::*kan*	(1)
bLH533	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-*yukE* (spec)	(1)
bLH530	*amyE*::Phyperspank-*yukE* (spec)	(1)
bTS033	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEW44A (spec)	This work
bTS040	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEW44G (spec)	This work
bTS041	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEW44Y (spec)	This work
bTS034	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEW44F (spec)	This work
bTS035	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEW44E (spec)	This work
bTS036	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEW44R (spec)	This work
bTS037	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEW44P (spec)	This work
bTS042	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEE45A (spec)	This work
bTS043	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEE45G (spec)	This work
bTS038	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEE45K (spec)	This work
bTS044	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEE45R (spec)	This work
bTS047	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEE45D (spec)	This work
bTS045	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEE45Q (spec)	This work
bTS039	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEG46A (spec)	This work
bTS049	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEG46P (spec)	This work
bTS048	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEG46R (spec)	This work
bTS125	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukET8C (spec)	This work
bTS126	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEK17C (spec)	This work
bTS127	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEV21C (spec)	This work
bTS129	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukES62C (spec)	This work
bTS131	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEQ75C (spec)	This work
bTS130	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEN82C (spec)	This work
bTS133	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukET8C/Q75C (spec)	This work
bTS134	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEK17C/N82C (spec)	This work
bTS135	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEK17C/S62C (spec)	This work
bTS136	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEV21C/S62C (spec)	This work
bTS188	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukECΔ1 (spec)	This work
bTS189	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukECΔ2 (spec)	This work
bTS058	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukECΔ3 (spec)	This work
bTS059	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukECΔ5 (spec)	This work
bTS060	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukECΔ7 (spec)	This work
bTS061	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukECΔ10 (spec)	This work
bTS062	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukECΔ12 (spec)	This work
bTS064	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukECΔ15 (spec)	This work
bTS109	*amyE*::Phyperspank-yukECΔ5 (spec)	This work
bTS110	*amyE*::Phyperspank-yukECΔ7 (spec)	This work
bTS111	*amyE*::Phyperspank-yukECΔ10 (spec)	This work
bTS112	*amyE*::Phyperspank-yukECΔ12 (spec)	This work
bTS114	*amyE*::Phyperspank-yukECΔ15 (spec)	This work
bTS241	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukECΔ5/S62C (spec)	This work
bTS242	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukECΔ7/S62C (spec)	This work
bTS243	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukECΔ10/S62C (spec)	This work
bTS245	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukECΔ15/S62C (spec)	This work
bTS282	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEW44A/S62C (spec)	This work
bTS283	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEE45A/S62C (spec)	This work
bTS284	*yukE*::*erm*-Pyuk; *amyE*::Phyperspank-yukEG46A/S62C (spec)	This work
E. coli		
DH5α	F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1	Invitrogen
BL21(DE3)	F– ompT hsdSB(rB–, mB–) gal dcm (DE3)	EMD
eTS026	BL21(DE3) pET28-his-yukE	This work
eTS279	BL21(DE3) pET28-his-yukECΔ5	This work
eTS283	BL21(DE3) pET28-his-yukECΔ15	This work

Table S2. Cont.

Name	Genotype	Source
eTS420	BL21(DE3) pET28-his-yukEW44A	This work
eTS421	BL21(DE3) pET28-his-yukEE45A	This work
eTS422	BL21(DE3) pET28-his-yukEG46A	This work

1. Huppert LA, et al. (2014) The ESX system in Bacillus subtilis mediates protein secretion. *PLoS ONE*, 10.1371/journal.pone.0096267.

Table S3. Oligonucleotides used in this study

Name	Sequence 5′→3′	Description	Source
Cloning *yukE* into pET28b(+) vector (Novagen)			
oTS010	agataacatatggcaggattaattcgtgtcac	Forward, NdeI	This work
oTS012	attggatccatactgttatccgcggatttg	Reverse, BamHI	This work
Mutagenesis of *B. subtilis* integration vector			
oTS130	cgcaaatcaaatccgctaaggataagctagccgca	yukECΔ1	This work
oTS131	caagacatcgcaaatcaaatctaacgcggataagctag	yukECΔ2	This work
oTS075	ccaagacatcgcaaatcaataagctagccgcatgcaag	yukECΔ3	This work
oTS076	cttgcatgcggctagcttattgatttgcgatgtcttgg	yukECΔ3	This work
oTS077	tgaccaagacatcgcataagctagccgcatgc	yukECΔ5	This work
oTS078	gcatgcggctagcttatgcgatgtcttggtca	yukECΔ5	This work
oTS079	gtctactgaccaagactaagctagccgcatgc	yukECΔ7	This work
oTS080	gcatgcggctagcttagtcttggtcagtagac	yukECΔ7	This work
oTS081	caaatacacttgagtctacttaagctagccgcatgcaagc	yukECΔ10	This work
oTS082	gcttgcatgcggctagcttaagtagactcaagtgtatttg	yukECΔ10	This work
oTS083	aacagcaaatacacttgagtaagctagccgcatgcaag	yukECΔ12	This work
oTS084	cttgcatgcggctagcttactcaagtgtatttgctgtt	yukECΔ12	This work
oTS087	tcagcagcttgatcaaacagcaaattaagctagccgcat	yukECΔ15	This work
oTS088	atgcggctagcttaatttgctgtttgatcaagctgctga	yukECΔ15	This work
oTS037	ctctgatttgaaaagcatggcggaaggtgcttcaagcgaa	yukEW44A	This work
oTS038	ctctgatttgaaaagcatgggcgaaggtgcttcaagcgaag	yukEW44G	This work
oTS039	ctctgatttgaaaagcatgtatgaaggtgcttcaagcgaag	yukEW44Y	This work
oTS040	ctctgatttgaaaagcatgttcgaaggtgcttcaagcgaag	yukEW44F	This work
oTS041	gatctctgatttgaaaagcatggaagaaggtgcttcaagcgaagcgt	yukEW44E	This work
oTS042	ctctgatttgaaaagcatgcgtgaaggtgcttcaagcgaag	yukEW44R	This work
oTS043	ctctgatttgaaaagcatgccggaaggtgcttcaagcgaa	yukEW44P	This work
oTS052	gatttgaaaagcatgtgggcgggtgcttcaagcgaagcgt	yukEE45A	This work
oTS053	gatttgaaaagcatgtggggcggtgcttcaagcgaagcgt	yukEE45G	This work
oTS054	gatttgaaaagcatgtggaaaggtgcttcaagcgaag	yukEE45K	This work
oTS055	tctgatttgaaaagcatgtggcgtggtgcttcaagcgaagcgttc	yukEE45R	This work
oTS056	gatttgaaaagcatgtggcagggtgcttcaagcgaagcg	yukEE45Q	This work
oTS057	gatttgaaaagcatgtgggatggtgcttcaagcg	yukEE45D	This work
oTS058	tgaaaagcatgtgggaagcggcttcaagcgaagcgttc	yukEG46A	This work
oTS059	tgatttgaaaagcatgtgggaaccggcttcaagcgaagcgttcg	yukEG46P	This work
oTS044	ggcaggattaattcgtgtctgccccgaagagctaagagcga	yukET8C	This work
oTS045	gagctaagagcgatggcgtgccaatacggcgttgaaagc	yukEK17C	This work
oTS046	agcgatggcgaagcaatacggctgcgaaagccaagaagtattaaatc	yukEV21C	This work
oTS049	gatcaatacgagcagctcaaaccttgctttatcaaaatgtcagatttgcttc	yukES62C	This work
oTS050	atgtcagatttgcttcaagatgtgaattgccagcttgatcaaacagcaaatacac	yukEQ75C	This work
oTS051	aatcagcagcttgatcaaacagcatgcacacttgagtctactgaccaag	yukEN82C	This work
Mutagenesis of *E. coli* expression vector			
oTS137	ctgaccaagacatcgcataaaatcaaatccgcggata	yukECΔ5	This work
oTS141	aatcagcagcttgatcaaacagcaaattaaacacttgagtctac	yukECΔ15	This work